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14. ABSTRACT

Cyclin E overexpression occurs in 25% of breast cancer tumors and is linked to poor prognosis. In tumor cells full length cyclin E (FL-E) is processed by an elastase-like protease into low-molecular weight isoforms (LMW-E) that are biochemically hyperactive. We recently demonstrated in a transgenic mouse model that CDK2 is required for LMW-E-induced breast cancer. The hypothesis is that the biological and biochemical differences between FL-E and LMW-E may be due to the phosphorylation of a distinct set of substrates when complexed with CDK2. The Protoarray analysis led us to discover Hbo1 and CINP as novel substrates of the LMW-E/CDK2 complex that may mediate critical downstream signaling to contribute to the oncogenic potential of LMW-E in breast cancer. We will pursue the identification of new substrates by phosphorylating a cell lysate in vitro with cyclin EL/CDK2 (F80G) and cyclin E-LMW/CDK2 (F80G) and PE-ATP-γ-S. The identification of new physiological LMW-E/CDK2 substrates will lead to the development of novel targets for therapeutics and the identification of the biological function for the treatment of the aggressive LMW-E expressing triple negative breast cancer.

15. SUBJECT TERMS

Cyclin E, CDK2, Breast Cancer, Cell Cycle

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Introduction:

Cyclin E overexpression occurs in 25% of breast cancer tumors and is linked to poor prognosis (Keyomarsi et al., 2002). In tumor cells full length cyclin E (FL-E) is processed by an elastase-like protease into low-molecular weight isoforms (LMW-E) that are biochemically hyperactive (Akli et al., 2004). We recently demonstrated in a transgenic mouse model that CDK2 is required for LMW-E-induced breast cancer and that CDK2 inhibitor (such as roscovitine) also delays mammary tumor formation (Akli et al., 2011). The hypothesis is that the biological and biochemical differences between FL-E and LMW-E may be due to the phosphorylation of a distinct set of substrates when complexed with CDK2. Our goal is to identify potential LMW-E/CDK2 substrates on a proteome-wide scale that could serve as novel therapeutic targets for the treatment of the aggressive LMW-E expressing triple negative breast cancer.

Body:

To identify potential human cyclin EL/CDK2 and LMW-E (T1)/CDK2 substrates, we first use ProtoArray Human Protein Microarray from Invitrogen containing more than 9,000 kinase substrates expressed as N-terminus GST fusion (Figure 1). Recombinant EL/CDK2 and LMW-E/CDK2 complexes were expressed and purified from insect cell lysates and the kinase assay was performed using GST-Rb as substrate to confirm that these complexes have active kinase activity (Figure 1A and 1B). Arrays were incubated either with recombinant active cyclin EL/CDK2 or cyclin E-LMW/CDK2 at a concentration of 50 nM in the presence of (γ-33P)-ATP for 1 hour at 30C. After washing and drying, arrays were exposed to X-ray film overnight. Imaging and data analysis were performed as recommended by the manufacturer. The radioactive signals were directly compared to generate a list of proteins that were most differentially phosphorylated by EL/CDK2 and LMW-E/CDK2 complexes (Figure 1C). Our screen identified a total of 146 potential substrates to both EL/CDK2 and LMW-E/CDK2 complexes. Interestingly, we only identified 4 proteins that were phosphorylated by EL/CDK2 significantly more than by LMW-E/CDK2 as compared to the 14 potential substrates that were preferentially phosphorylated by LMW-E/CDK2 suggesting that by losing the N-terminal portion, the LMW-E/CDK2 kinase complex is able to specifically interact and phosphorylate additional proteins (Figure 1D and Table 1).

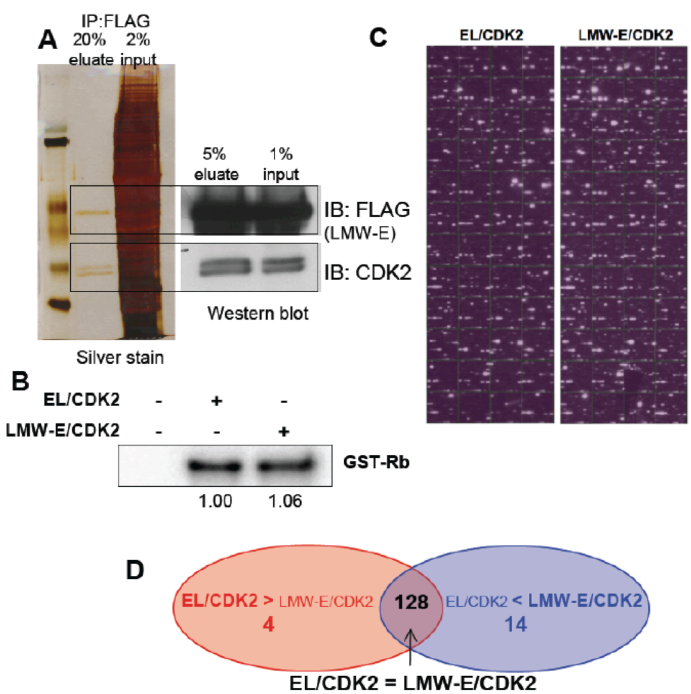

Figure 1: Identification of human EL/CDK2 and LMW-E/CDK2 substrates using the ProtoArray microarray. (A) FLAG-EL/CDK2 and FLAG-LMW-E/CDK2 complexes were expressed in sf9 insect cells, purified by IP with FLAG-tagged antibody, eluted with 3X FLAG peptide and visualized by silver stain and Western blot analysis. (Only LMW-E/CDK2 results are shown here). (B) *In vitro* kinase assay using purified EL/CDK2 and LMW-E/CDK2 kinase complexes with GST-Rb as substrate to confirm the relative amount of the kinase complexes for use in the microarray analysis. The kinase assay was performed with 32P-γ-ATP, separated by SDS-PAGE and exposed to x-ray films. (C) The microarrays were incubated either with recombinant EL/CDK2 or LMW-E/CDK2 in the presence of 33P-γ-ATP and the radioactive signals were exposed to x-ray films. (D) Venn diagram showing the number of proteins whose phosphorylation signal by EL/CDK2 is greater than LMW-E/CDK2 by more than 1.5 (red), LMW-E/CDK2 signal is greater than EL/CDK2 signal by 1.5 (blue) and EL/CDK2 and LMW-E/CDK2 signals are between 0.5 and 1.5 (black).

In our list of 14 potential substrates preferentially phosphorylated by LMW-E/CDK2, we chose 2 proteins for validation. Hbo1 (histone acetyltransferase (HAT) binding to ORC1 (origin recognition complex 1)) and CINP (Cdk2-Interacting Protein) were phosphorylated by LMW-E/CDK2 with phosphorylation signal 3.5-fold and 7.5-fold higher than when phosphorylated by EL/CDK2 (Figure 2A). Hbo1 has been implicated in regulating gene expression, DNA replication, and DNA repair and is proposed as a potential oncogene in breast cancer (Iizuka et al., 2009). CINP was identified as a regulator of ATR-mediated checkpoint signaling (Lovejoy et al., 2009). CINP promotes cell viability in response to replication stress, is required for efficient ATR-dependent signaling after DNA damage, and is required for maintenance of the G2 checkpoint. Since Hbo1 could be a mediator of LMW-E-induced changes in gene expression and CINP, a mediator of LMW-E-induced genomic instability, we performed further experiments with these 2 proteins.

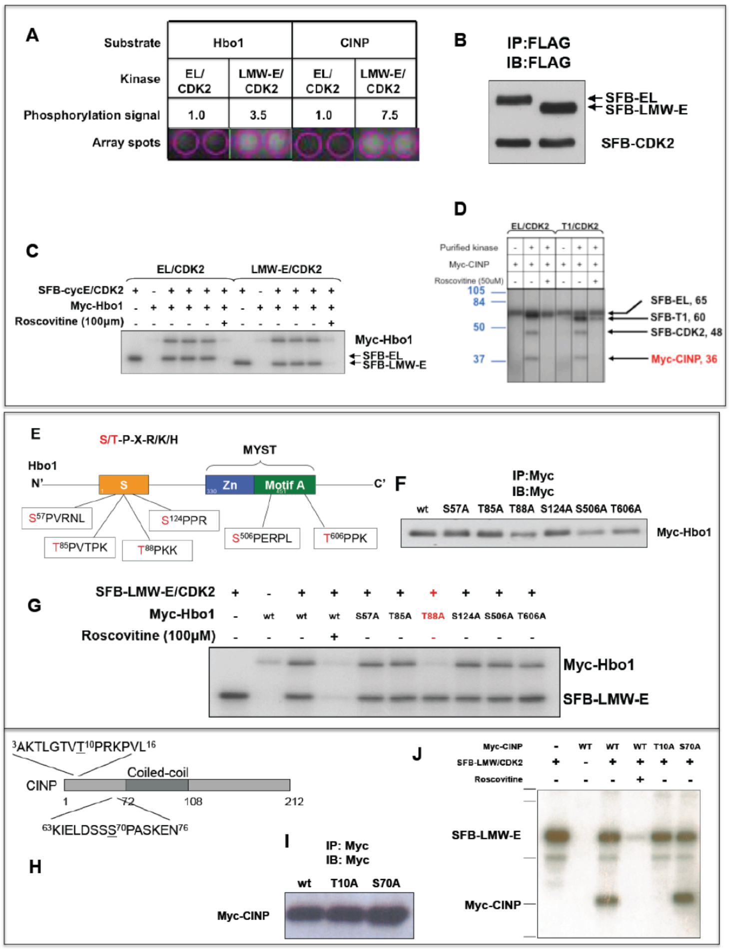

Figure 2: Hbo1 and CINP are novel substrates of the cyclin E/CDK2 complex. (A) The ProtoArray microarray experiment showing Hbo1 and CINP spots. The phosphorylation signals indicate relative radioactive signal detected in the microarray spots. (B) Triple epitope tag Streptavidin-Flag-S peptide SFB-EL or SFB-LMW-E was co-transfected with SFB-CDK2 into HEK293T cells, purified using FLAG-tagged antibody, eluted with 3X FLAG peptide and visualized by Western blot analysis. Myc-Hbo1 and Myc-CINP were transfected into HEK293T cells, purified using Myc-tagged antibody and the bead-Myc protein complex was resuspended in 1X wash buffer. The EL/CDK2 or LMW-E/CDK2 kinase complex was incubated with purified Hbo1 (C) or purified CINP (D) in the presence of ^{32}P-γ-ATP and with or without roscovitine. The samples were separated by SDS-PAGE and exposed to x-ray films. (E) Schematic of the Hbo1 coding sequence with the potential phosphorylation sites predicted based on the CDK2 consensus phosphorylation motif (S/T-P-X-R/K/H). (F & G) The six potential phosphorylation sites were mutated to alanine, expressed, purified and subjected to similar kinase assay as in (C). (H) Schematic of the CINP coding sequence with the potential phosphorylation sites. (I & J) The 2 potential phosphorylation sites were mutated to alanine, expressed, purified and subjected to similar kinase assay as in (D).

To confirm whether Hbo1 and CINP are substrates of the cyclin E/CDK2 kinase complex, the EL/CDK2 and LMW-E/CDK2 kinase complexes and Myc-Hbo1 and Myc-CINP proteins were purified by IP with FLAG-tagged and Myc-tagged antibodies, respectively (Figure 2B). Results from the *in vitro* kinase assay showed that both EL/CDK2 and LMW-E/CDK2 kinase complexes phosphorylate Hbo1 at relatively similar levels, and addition of roscovitine efficiently inhibited the Hbo1 phosphorylation signal (Figure 2C). Similar results were obtained with CINP (Figure 2D). Based on the consensus CDK2 phosphorylation motifs (S/T-P-X-R/K/H and R-X-L), there are six potential CDK2 phosphorylation sites on the Hbo1 protein coding sequence (Figure 2E), and 2 potential CDK2 phosphorylation sites on the CINP protein coding sequence (Figure 2H). These sites were mutated to alanine to identify which site is being phosphorylated by the LMW-E/CDK2 complex. The mutant proteins were transfected into HEK293T cells, purified by immunoprecipitation followed by kinase assay (Figure 2F-G for Hbo1 and 2 I-J for CINP). Of the six potential sites, the LMW-E/CDK2 complex phosphorylates Hbo1 at T88 since the T88A mutant showed abolished radioactive signal (Figure 2G). Of the 2 potential sites, the LMW-E/CDK2 complex phosphorylates CINP at T10 since the T10A mutant showed abolished radioactive signal (Figure 2J). Collectively, the Protoarray analysis led us to discover Hbo1 and CINP as novel substrates of the LMW-E/CDK2 complex that may mediate critical downstream signaling to contribute to the oncogenic potential of LMW-E in breast cancer.

The second approach for LMW-E-CDK2 substrate identification is a chemical/genetic approach in which an analog sensitive CDK2 kinase, (F80A or F80G)-CDK2 is used to specifically radiolabel its substrates in cell extracts followed by their identification by mass spectroscopy.

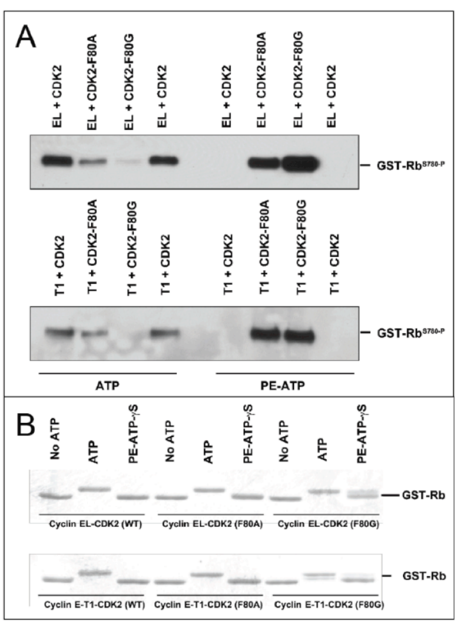

Figure 3: Characterization of the engineered CDK2. (A) Cyclin EL and LMW-E (T1)-CDK2 or their F80G and F80A engineered complex counterparts were purified from insect cells by affinity purification/elution with FLAG and subjected to GST-Rb in vitro kinases assays with either normal ATP or PE-ATP analogue, followed by western blot with anti-pS780-Rb antibody. The wild-type kinase cannot use PE-ATP. (B) GST-RB kinase assays using wild type and cyclin EL- or T1-CDK2 (F80A) or (F80G) complexes in the presence of ATP and PE-ATP-g-S. Kinase reactions were analysed by SDS-PAGE and visualized by Coomassie staining. Extend of the GST-Rb phosphorylation was monitored by the electromobility shift of GST-Rb.

The two CDK2 mutants generated are the phenylalanine (F) to alanine (A) and a F to glycine (G) exchange at position 80, designated CDK2 (F80A) and CDK2 (F80G). We then determined if these 2 engineered CDK2s could use 2 ATP analogues, PE-ATP and PE-ATP-γ-S to phosphorylate pRb *in vitro*. We found that although both wild-type and cyclin E/CDK2 (F80A) and to a lesser extend cyclin E/CDK2 (F80G) used normal ATP to phosphorylate GST-Rb protein, only F80A and F80G kinases could use PE-ATP (Figure 3A). We then expressed and purified wild-type CDK2 in complex with cyclin EL or cyclin E-LMW and CDK2 (F80A) and CDK2 (F80G) from insect cells and carried out a similar

Rb kinase assay to test their ability to use PE-ATP-γ-S. As shown in figure 3B, although all 3 CDK2 kinases can use ATP to phosphorylate GST-Rb protein as indicated by its electromobility shift, only the F80G mutant can use PE-ATP-γ-S. We will use the F80G mutant for all our subsequent experiments. The next steps will be to phosphorylate a cell lysate in vitro with cyclin EL/CDK2 (F80G) and cyclin E-LMW/CDK2 (F80G) and PE-ATP-γ-S. The protein mixture will be digested, the thiophosphopeptides will be captured with thiopropyl sepharose and the thiophosphopeptides will be specifically released by treatment of the resin with a strong base. The recovered peptides will be subjected to liquid chromatography-MS/MS analysis to identify and to compare the substrates phosphorylated by LMW-E/CDK2 and FL-E/CDK2 kinase.

Key Research Accomplishments:

1. Identification of a total of 146 potential substrates to both EL/CDK2 and LMW-E/CDK2 complexes using the ProtoArray Human Protein Microarray from Invitrogen including 14 potential substrates that were preferentially phosphorylated by LMW-E/CDK2.

2. *In vitro* validation of Hbo1 and CINP as new LMW-E-CDK2 substrates.

3. Identification of T88 as the LMW-E/CDK2 phosphorylated site on Hbo1 and T10 as the LMW-E/CDK2 phosphorylated site on CINP.

4. Expression and purification of wild-type CDK2, CDK2 (F80A) and CDK2 (F80G) in complex with cyclin E-EL or cyclin E-LMW from insect cells.

5. Demonstration that cyclin E-LMW/CDK2 (F80G) efficiently use PE-ATP-γ-S to phosphorylate GST-Rb in an in vitro kinase assay.

Reportable Outcomes:

Presented poster entitled “Identification of new substrates for breast tumor specific low-molecular-weight cyclin E cyclin-dependent-kinase 2” at the Era of Hope meeting in Orlando FL, 2-5 Aug. 2011

Submission of a paper entitled “Hbo1 is a novel substrate of LMW-E/CDK2 and enriches for the cancer stem cell population in breast cancer” reporting some of the data generated by this award.

Conclusions:

The Protoarray analysis led us to discover Hbo1 and CINP as novel substrates of the LMW-E/CDK2 complex that may mediate critical downstream signaling to contribute to the oncogenic potential of LMW-E in breast cancer.

Hbo1 may be the mediator of LMW-E-induced changes in gene expression leading to the enrichment of the cancer stem cell population in breast cancer.

The requirement for CINP T10 phosphorylation in the resistance to replication stress and in G2 checkpoint maintenance, two checkpoint functions compromised by silencing of CINP will be examined in a variety of breast cancer cell lines.

We will pursue the identification of new substrates by phosphorylating a cell lysate in vitro with cyclin EL/CDK2 (F80G) and cyclin E-LMW/CDK2 (F80G) and PE-ATP-γ-S. The protein mixture will be digested, the thiophosphopeptides will be captured with thiopropyl sepharose and the thiophosphopeptides will be specifically released by treatment of the resin with a strong base. The recovered peptides will be subjected to liquid chromatography-MS/MS analysis to identify and to compare the substrates phosphorylated by LMW-E/CDK2 and FL-E/CDK2 kinases.

The identification of new physiological LMW-E/CDK2 substrates will lead to the development of novel targets for therapeutics and the identification of the biological function for the treatment of the aggressive LMW-E expressing triple negative breast cancer.

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